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13. ABSTRACT ***(Maximum 200 Words)*** Two drugs have been studied as potential therapeutic agents for late stage breast cancer patients. 5-aza-2'deoxycytidine (5-Aza-CdR), is an inhibitor of DNA methylation and is targeted for re-expression of a repressed tumor suppressor gene known as p16. Re-activation of the p16 gene in cells in which the gene is methylated will restore normal growth control and be efficacious in treatment of breast cancer patients. Both p16 protein expression and de-methylation of the p16 promoter occurs in breast cancer cells, treated with 5-Aza-CdR. 5-Aza-CdR also destroys the ability of the cells to grow in an anchorage-dependent manner, thereby indicating that the drug can inhibit tumorigenesis. We are currently developing a better model for the action of the drug by screening a number of breast cancer cell lines for methylated p16 and then testing the efficacy of the drug in a nude mouse model. A non-invasive blood-test has been developed for the detection of p16 methylation that will allow us to easily identify patients suitable for a 5-Aza-CdR clinical trial. Clinical protocols to accomplish this goal have been submitted. A phase II clinical trial with a second drug, Bryostatin-1, will continue but has been temporarily halted due to administrative problems.

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Introduction

During the past decade a very large increase in our knowledge of biological mechanisms regulating progression of cells through the cell division cycle has taken place. Together with the development of this knowledge, it has been shown by work in many laboratories that essentially all cancer cells have one or more defects in the components known to regulate cell cycle progression. For example, our recently completed studies of cell cycle regulatory defects in breast cancer cells, carried out with a grant from the Army Breast Cancer Research Program, showed that loss of expression of the cyclin-dependent kinase inhibitor p16, sometimes accompanied by overexpression of cyclin D1, is a common defect in breast cancer cells. These findings, plus the large amount of work carried out by others, presented a new potential target for cancer chemotherapy. Our proposal to exploit such targets for the chemotherapy of breast cancer is the basis for the current Clinical Translational Research Grant. We proposed to explore two drugs known or expected to cause changes in the expression of cell cycle regulatory components as potential chemotherapeutic agents in the treatment of late stage breast cancer. Bryostatin-1, shown by Kraft and coworkers to cause increases in the expression of the cyclin-dependent kinase inhibitor p21, was chosen as an agent to be tested in a phase II clinical trial. 5,6-dihydro-5-azacytidine, a DNA methylation inhibitor with less toxicity than the commonly studied 5-aza-2'-deoxycytidine, was chosen for pre-clinical studies directed towards eventually implementing a phase II clinical trial of that drug. DNA methylase inhibitors have been shown to increase the expression of p16 protein in cells where lack of expression is due to methylation of the p16 gene. The results of the second year of the grant are reported here. These results indicate that some modifications in the approaches originally proposed may be desirable to best achieve the goals of the grant.

Body of Report

Materials and Methods

Breast cancer cell lines and tumor material

The breast cancer cell lines MCF-7, T47-D and ZR75.1 were obtained from the University of Colorado Tissue Culture Core Facility and the breast and colon cancer cell lines, DU4475 and HCT-15, respectively, were obtained from the American Type Culture Collection. DU4475 was cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 0.2% sodium bicarbonate, 18 mM HEPES, 1% non-essential amino acids, 1 mM sodium pyruvate and 2 mM L-glutamine. HCT-15 was cultured in MEM media supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 1 mM non-essential amino acids and 2 mM L-glutamine. MCF-7 and ZR75.1 were cultured in MEM media supplemented with 10% fetal calf serum, 0.2% sodium bicarbonate, 10 mM HEPES, 1% non-essential amino acids, 2 mM L-glutamine and 6 ng/ml insulin.

Antibodies

The anti-p16 was obtained from Oncogene. The horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad.

Treatment of cells with 5-Aza-CdR

Cells were plated into 10 cm dishes and allowed to grow to 30-40% confluence before the addition of the appropriate concentration of 5-Aza-CdR. Cells were incubated at 37°C for the desired period of time and provided with fresh media plus or minus the appropriate concentration of 5-Aza-CdR twice per week.

Protein extraction and western blot analysis

Cells were harvested and washed in PBS then resuspended in Laemmli sample buffer (Laemmli., 1970). After boiling for 4 minutes, the extracts were sheared through a 26-gauge syringe needle, aliquoted, and stored at -80°C.

Approximately 100 μg of each protein extract were subjected to SDS/PAGE (Laemmli., 1970) and transferred to nitrocellulose membranes (Schleicher and Schuell) for 45 minutes at 0.45 A using the Genie Electrophoretic Blotter (Idea Scientific, Minneapolis). Membranes were stained with Ponceau dye to control for equal loading, and immunodetection performed using the enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

Isolation of DNA from cell lines and blood plasma

Cell line DNA was isolated by incubating cells at 55°C in lysis buffer (10 mM Tris pH 8.0, 2.0 mM EDTA pH 8.0, 10 mM NaCl, 5% SDS) containing 1 mg/ml Proteinase K. The samples were then subjected to two phenol-chloroform extractions and one chloroform:isoamylalcohol (24:1) extraction followed by ethanol precipitation.

To isolate plasma DNA, 10 ml plasma were first heated to 99°C for 5 minutes then centrifuged at 14 K rpm. The clear supernatant was incubated overnight in one-tenth volume of 20 mg/ml Proteinase K (in double distilled water) and one-tenth volume AL buffer (Qiamp Blood Kit, Qiagen Inc., Hilden, Germany) after which the DNA was purified on QIAamp columns (Qiamp Blood Kit, Qiagen Inc., Hilden, Germany) according to the 'Blood and Body Fluids protocol'. The DNA was eluted from the column with 200 µl double distilled water.

DNA analysis by Methylation-specific PCR (MSP)

Two micrograms of cell line DNA or one-fourth of the total plasma DNA sample were modified with sodium bisulfite using a modified method of Herman et al. (1996) kindly sent to us by S. Belinsky (University of New Mexico), and precipitated with ammonium acetate (3M final concentration) and two volumes of ethanol. The resulting templates were subjected to PCR using oligonucleotides designed from the promoter of the p16 gene (Herman et al., 1996) specific for wildtype, methylated or unmethylated DNA using our own modification of the method of Herman et al. (1996). A 20 µl reaction mixture overlaid with a drop of mineral oil contained a final concentration of 120 ng of each oligonucleotide, PCR buffer supplied by Promega (10 mM tris-HCL, pH 8.3; 5 mM KCl; 0.1% gelatin); 200 µM dNTPs; 1.5 mM $MgCl_2$; and 0.06 units μl^{-1} Taq polymerase (added once the reaction temperature reached 95°C). The DNA was subjected to 35 cycles of amplification (for cell line DNA) or 55 cycles of amplification (for plasma DNA) consisting of denaturation for 0.5 minutes at 94°C, annealing for 0.5 minutes at 60°C (for unmethylated-specific oligonucleotides) or 65°C (for wildtype- and methylated-specific oligonucleotides), and elongation for 0.5 minutes at 72°C, followed by a final elongation step of 10 minutes at 72°C. The PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized with uv light.

Soft agar analysis

Five milliliters of a mixture containing 0.4% agar, 10% serum, 1 X MEM (for T47-D, MCF-7) or 1 X RPMI (for DU4475) medium, plus or minus 1 µM 5-Aza-CdR (previously incubated at 45°C) were added to a specified number of 60 mm plates, and allowed to harden at room temperature. Each of the cell lines, that had been grown in the presence or absence of 1 µM 5-Aza-CdR in two 75 cm^2 flasks for 4 days, were harvested and counted. The cells were resuspended in the agar/medium mixture described above to a specified concentration and plated on to the 60 mm dishes containing the base layer of

agar/medium. The dishes were incubated at 37°C and supplemented with fresh media plus or minus 1 μM 5-Aza-CdR twice per week.

Results

I. Induction of p16 expression in breast cancer cell lines following exposure to 5'-Aza-2'-Deoxycytidine.

In the 1999 Progress Report, we described the optimization of 5'-Aza-2'-Deoxycytidine (5-Aza-CdR)-mediated induction of p16 expression in the HCT-15 colon cancer and T24 bladder cancer cell lines, respectively. These two cell lines were previously reported to be transcriptionally silenced at the p16 locus due to promoter methylation but to undergo reexpression of p16 in response to continuous exposure to the demethylating agent, 5-Aza-CdR at concentrations of 0.1 μM or 1 μM for 9 days (Bender et al., 1998). Our experiments demonstrated that treatment of the cells with 1 μM 5-Aza-CdR for as little as 5 days also resulted in a marked induction of p16 protein expression as assayed by western blot.

Our next objective was to determine the minimal length of exposure necessary for 5-Aza-CdR to induce p16 protein expression in breast cancer cell lines that lacked endogenous p16 due to promoter methylation. The T47-D and ZR75.1 breast cancer cell lines were chosen for analysis as they lacked endogenous p16 protein but, importantly, expressed functional RB protein, the latter being required to mediate a G1 growth arrest in response to p16 function. As a negative control, we employed the MCF-7 breast cancer cell line, which expressed RB but was homozygously deleted at the p16 locus. The HCT-15 colon cancer cell line which we previously showed to undergo 5-Aza-CdR-mediated reexpression of p16 protein, was employed as the positive control. In addition, the breast cancer cell line, DU4475, was included in the analysis as it lacked both p16 – due to promoter methylation - and RB protein expression. Thus, in the event that p16 was induced by 5-Aza-CdR in this cell line, the absence of endogenous RB protein would prevent p16-dependent effects upon cell growth.

Logarithmic phase cultures of each of the cell lines were treated continuously with 1 μM 5-Aza-CdR for different lengths of time ranging from 0-10 days at 37°C. Protein was isolated from both the untreated and treated cells and assayed for p16 expression by western blot. Owing to the homozygous deletion at the p16 locus, we detected no induction of p16 expression in the MCF-7 cell line. In contrast, HCT-15 cells showed a positive correlation between the duration of 5-Aza-CdR treatment and degree of p16 induction with initial detection of the protein occurring at two days post treatment (Figure 1). T47-D cells underwent induction of p16 expression following at least 3 days continuous exposure to 5-Aza-CdR with significantly elevated induction of p16 following 5 days exposure to 5-Aza-CdR (Figure 1). DU4475 cells also underwent induction of p16 expression in the presence of 5-Aza-CdR (Figure 1). However, unlike the T47-D and HCT-15 which showed increased expression with increased duration of drug treatment, the level of p16 induction in the DU4475 cells remained constant following 4, 8 and 10 days exposure to 5-Aza-CdR (Figure 1). Furthermore, the degree of induction was not

elevated following the treatment of the cells with even higher concentrations and/or length of exposure to 5-Aza-CdR (data not shown). We observed no detectable induction of p16 expression in the ZR75.1 breast cancer cell line even after exposure to 5 and 10 μM 5-Aza-CdR for up to 14 days (data not shown). Thus, at this point, we removed the ZR75.1 cell line from our study.

II. 5-Aza-CdR-mediated induction of p16 expression is preceded by demethylation of the p16 gene promoter in breast cancer cell lines.

To determine if the 5-Aza-CdR-mediated induction of p16 protein expression exhibited by the T47-D, DU4475 and HCT-15 cell lines was a consequence of promoter demethylation, we isolated DNA from both the untreated and treated cell lines, described above, and subjected it to methylation-specific PCR (MSP) (Herman et al., 1996). Briefly, the DNA was treated with sodium bisulfite (which converts all unmethylated cytosines to uracil but does not alter methylated cytosines), purified and subjected to PCR analysis using primers specific for either wildtype (W), methylated (M) or unmethylated (U) p16 promoter sequences (Herman et al., 1996). DNA derived from untreated and treated MCF-7 cells was also assayed and, as expected, showed no amplification with any of the p16-specific primers (Figure 2). DNA isolated from untreated T47-D, HCT-15 and DU4475 cells contained only methylated p16 (Figure 2). However, DNA derived from 5-Aza-CdR-treated cells underwent amplification using primers specific for both methylated and unmethylated p16, consistent with the induction of p16 protein expression in these 3 cell lines (Figure 2). Notably, the demethylation of the p16 promoter was evident by day 2 in the T47-D cells whereas expression of the p16 protein was not detected until day 3 post-drug treatment (Figure 2). These data confirm that the reexpression of p16 protein was a direct consequence of the demethylation of the p16 promoter by 5-Aza-CdR.

III. 5-Aza-CdR treatment inhibits the ability of breast cancer cell lines to grow in an anchorage-independent manner.

Unlike normal cells which require adherence to a substratum in order to divide and proliferate, tumor cells grow in an anchorage-independent manner. The mechanisms underlying this particular tumor-specific loss of growth control have yet to be fully elucidated but are likely to involve cell cycle checkpoint deregulation. Indeed, we have previously reported that relatively modest levels of p16 protein expressed under the control of an inducible 'Tet' promoter will suppress the growth of MCF-7 breast cancer cells in soft agar (Todd et al., manuscript in preparation).

To assay the effect of 5-Aza-CdR treatment upon the ability of breast cancer cells to grow in the absence of anchorage to a substratum, we subjected the T47-D, DU4475 and MCF-7 cell lines to soft agar analysis following culturing in the presence or absence of 5-Aza-CdR. Duplicate cultures of each of the three cell lines were grown in two 75 cm^2 flasks, one of which contained medium supplemented with 1 μM 5-Aza-CdR, for 4 days at 37°C, thus allowing sufficient time - based upon the data from the experiments described above - for p16 promoter demethylation and protein expression to occur. The

cells were then harvested for the parallel analyses of p16 expression and growth in soft agar.

As expected, both the T47-D and DU4475 cells showed induction of p16 protein expression after exposure to 5-Aza-CdR for 4 days (Figure 3), while the MCF-7 cells that harbored a homozygous deletion at the p16 locus failed to show induction of p16 expression (Figure 3).

Cells grown in the absence of 5-Aza-CdR (-AZA) were plated onto each of 4 soft agar dishes lacking 5-Aza-CdR at 2.5 $x10^5$ cells/dish. Cells grown in the presence of 5-Aza-CdR (+AZA) were plated onto each of 8 soft agar dishes, four of which lacked 5-Aza-CdR and 4 of which contained 1 μM 5-Aza-CdR, at 2.5 x 10^5 cells/dish. The plating of drug-treated cells onto soft agar in the presence or absence of 5-Aza-CdR enabled us to determine if there was a continuous requirement for 5-Aza-CdR in the agar/medium in order to see a sustained effect (if any) upon the growth of cells in soft agar. Fresh media plus or minus 5-Aza-CdR (as appropriate) was added to the dishes twice per week and after approximately 2 weeks the number of colonies per dish were counted (Table 1) and representative dishes photographed (Figure 3).

All three of the cell lines cultured in the absence of 5-Aza-CdR (-AZA) grew very well in soft agar to form many large, healthy colonies, as indicated in Figure 3 and Table 1. In contrast, those cells cultured in the presence of 1 μM 5-Aza-CdR (+AZA) and maintained either in the presence or absence of 5-Aza-CdR in soft agar were significantly growth compromised with the vast majority failing to undergo cell division while a tiny minority underwent a maximum of 3-4 cell divisions before arresting (Figure 3 and Table 1). This effect on growth in soft agar is in contrast to the absence of significant effects of 5-Aza-Cdr on these cells when grown attached to substratum in standard culture dishes, indicating a specific effect on anchorage-independence. These data suggested that the restoration of the anchorage-dependent phenotype in these cell lines was attributable to the effects of the demethylating agent, 5-Aza-CdR. Notably, the DU4475 cells cultured in the presence of 5-Aza-CdR but plated onto soft agar lacking 5-Aza-CdR appeared to have a slight growth advantage over those maintained in the continuous presence of the drug. This lends further support to the role of 5-Aza-CdR in the inhibition of anchorage-independent growth in this cell line. These experiments have been repeated at least once for each cell line and have yielded reproducible results.

These data indicated that the 5-Aza-CdR was capable of suppressing the tumor-specific anchorage-independent properties of each of the three breast cancer cell lines described above. Had this marked growth inhibition been demonstrated by the T47-D cell line alone, we would have concluded a distinct role for p16 in the restoration of anchorage-dependence. However, the finding that both the MCF-7 cells (which were homozygously deleted at the p16 locus) and DU4475 cells (which lacked endogenous RB, an essential downstream mediator of p16 function) were also severely growth inhibited in response to 5-Aza-CdR treatment suggested the involvement of loci, other than p16, whose reactivation resulted in the suppression of the anchorage-independent phenotype in these two cell lines. Because the demethylating effects of 5-Aza-CdR are

not locus-specific, it is highly likely that treatment of the three breast cancer cell lines resulted in the demethylation and consequent reexpression of multiple genes that had been previously rendered 'transcriptionally silent' by de novo methylation. These findings do not rule out a role for p16 in regulation of anchorage-dependence but instead, indicate the involvement of more than one locus or genetic pathway in the regulation of this most fundamental growth control mechanism. Importantly, it appears that at least one of the genes reactivated by 5-Aza-CdR was involved in the suppression of anchorage-independence demonstrated by the three breast cancer cell lines, suggesting a potential role for this drug in breast cancer therapy.

We are currently in the process of screening additional breast cancer cell lines with the aim of identifying those which, like T47-D, express functional RB protein but lack p16 expression due to methylation. All cell lines that satisfy this criteria will be assayed for reexpression of p16 protein in response to 5-Aza-CdR treatment and subjected to soft agar analysis to determine the effectiveness of 5-Aza-CdR is suppressing the anchorage-independent phenotype in a larger sample number of breast cancer cell lines. In addition, we plan to determine the effect of 5-Aza-CdR treatment upon the in vivo tumorigenicity of breast cancer cell lines. Breast cancer cells will be injected in nude mice and allowed to form palpable tumors at which point 5-Aza-CdR will be administered. The tumors will then be measured biweekly to determine if the presence of the demethylating agent is capable of suppressing or causing a regression of tumor growth.

IV. Analysis of plasma DNA for breast tumor-specific methylation of the p16 gene.

Several years ago, it was determined that the plasma component of circulating blood contained tiny quantities of free DNA. The concentration of DNA in the plasma of healthy individuals is approximately 14 ng/ml (Shapiro et al, 1983). This level increases significantly in individuals diagnosed with different types of cancer to approximately 180 ng/ml (Leon et al., 1977).

The ability to detect tumor-specific molecular defects in the circulating blood would obviate the requirement for tumor biopsy material thus providing a more efficient and non-invasive means of screening for a vast array of molecular aberrations. One of the major objectives of the current project is to identify women whose tumors contain methylated p16 genes for recruitment into a clinical trial that aims to test the efficacy of therapeutic demethylating agents. In an attempt both to improve the efficiency and reduce the patient discomfort associated with screening, we have optimized the procedure for isolating DNA from normal plasma samples (a modification of that described by Silva et al., 1999) and shown that the resulting DNA can be successfully amplified at the p16 locus by methylation-specific PCR (MSP) (Herman et al., 1996).

V. Optimization of DNA isolation from frozen plasma.

A 10 ml and a 20 ml aliquot of frozen plasma derived from the same healthy human donor were thawed, heated to 99°C for 5 minutes then centrifuged at high speed to

allow recovery of the clear, DNA-containing supernatant. Following an overnight incubation with Proteinase K, the DNA samples were purified on QIAamp columns according to the 'Blood and Body Fluids' protocol and eluted in final volumes of 200 µl with double distilled water. We then subjected 20 µl and 40 µl aliquots of each of the two purified DNA samples to modification with sodium bisulfite, the latter of which converts all unmethylated cytosines to uracil. Following column purification the DNA samples were precipitated with ammonium acetate and ethanol and one-tenth of each subjected to MSP using primers to wildtype (W), methylated (M) and unmethylated (U) p16 promoter sequences. The breast cancer cell lines DU4475 and MDA-MB-231 were employed as methylated and unmethylated controls, respectively. DNA derived from both the 20 µl and 40 µl modification reactions (from the original 10 ml and 20 ml plasma aliquots) underwent amplification with the unmethylated DNA-specific primers only, representative data of which is shown in Figure 4. These experiments have been repeated multiple times starting with 10 ml frozen plasma with reproducible results. These data demonstrated that this method was capable of detecting the exceptionally low levels of free, unmethylated p16 DNA found in the circulating blood plasma of healthy individuals.

VI. Detection of low levels of methylated p16 DNA titrated into normal plasma.

In order to approximate the minimal level of methylated p16 DNA that could be detected in plasma DNA by MSP, we titrated cell line DNA containing methylated p16 alleles into individual 10 ml aliquots of normal plasma. Specifically, 0, 10, 20, 50, 100, 250 and 500 ng of HCT-15 colon cancer cell line DNA were added to individual 10 ml aliquots of normal plasma (from the same donor as that used in the above experiments). After thoroughly mixing, DNA was isolated from the plasma samples as described above and subjected to MSP analysis. The HCT-15 cell line DNA was employed as a control for methylated p16 and a subclone of the T47-D breast cancer cell line was employed as a control for both methylated and unmethylated p16. As expected, DNA isolated from plasma lacking the addition of HCT-15 DNA underwent amplification with the unmethylated DNA-specific primers only (Figure 5). The same was true for the five plasma aliquots to which 10-250 ng of HCT-15 DNA was originally added (Figure 5). However, we observed strong amplification of both unmethylated- and methylated-specific p16, respectively, from the plasma to which 500 ng of HCT-15 was originally added (Figure 5). Because only one-fortieth of the total amount of purified plasma DNA was used in the actual MSP reactions, these experiments demonstrated that we can successfully amplify as little as 12.5 ng of methylated p16 DNA in addition to the unmethylated p16 found in normal plasma DNA using this method. Because the normal plasma DNA of breast cancer patients should contain only unmethylated p16 DNA, this system should enable us to efficiently identify those individuals with breast tumor-derived methylated p16 alleles. Thus, such an approach may prove a feasible alternative to biopsy analysis as a means of screening for tumor-specific molecular aberrations.

VII. Detection of methylated p16 DNA in serum of breast cancer patients.

In order to determine whether detection of methylated p16 sequences in DNA from plasma of breast cancer patients is possible with the procedures described above, we carried out preliminary analysis of plasma DNA isolated from seven patients with metastatic breast cancer. As shown in Figure 6, unmethylated p16 DNA was readily detected in all patients. In addition, in two of seven patients (#'s 3 and 5) methylated p16 DNA was detected, indicating the likelihood that breast tumor p16 sequences in these patients is methylated. The frequency of p16 methylation seen in this small sample corresponds well with the frequency of p16 gene methylation seen by us and others of roughly one-third in breast cancer tissue and cell lines. As described in the revised Statement of Work and below (Section VIII), we have written and are awaiting approval of clinical protocols designed to test whether detection of methylated p16 sequences in plasma DNA is a reliable indicator of breast tumor p16 gene methylation. These studies of p16 methylation in breast cancer patients have also shown that methylated p16 DNA is stable in plasma stored frozen for up to 9 years, and that as little as 2 ml of plasma is sufficient. This increases the utility of our assay and allows for both retrospective and prospective studies.

VIII. Implementation of a phase II clinical trial to test the chemotherapeutic efficacy of bryostatin 1 in stage IV breast cancer patients and our new clinical efforts.

Clinical Trial

During much of this year, the University of Colorado Health Sciences Center clinical investigations has been under suspension by the FDA. This suspension was due to poor record keeping and not patient related activities. When the suspension was listed all protocols were resubmitted to the local IRB for reapproval. This process has been completed for the bryostatin-1 protocol. In addition, the Division of Medical Oncology has recruited a breast cancer expert from Harvard, Dr. Anthony Elias, who will work with Dr. Kraft to place patients on this trial.

New Clinical Efforts

The next clinical stage of this grant calls for the development of a clinical protocol using agents that inhibit the methylation of DNA in clinical trials. The rationale behind such a protocol is that the p16 gene is methylated in approximately 30% of breast cancer cases (Figure 6). Inhibition of DNA methylation would allow the synthesis of this protein and inhibit the cell cycle.

Before beginning to develop such a protocol it would be key to identify individuals that have this methylation. These individuals would be a select population that would stand to benefit from this study. It would be possible to assay patient tumor tissue at the time of operation for this methylation, but that is cumbersome and many times difficult. Referred patients would not have tumor tissue available.

To overcome this difficulty we have developed an assay based on a plasma sample that measures the extent of methylated p16 DNA in the blood (Figure 6). This p16 DNA in the blood would occur from tumor cell turnover and death.

To validate this assay we have developed two clinical protocols to obtain blood samples from patients (see Appendix). The goal of these studies is simply to validate our plasma assay only. Laboratory work continues on methylation-inhibition therapy in tissue culture and animals.

The first protocol entitled, "Correlation between methylated p16 DNA in the blood and breast cancer tissue of patients" will assay methylated p16 in breast tumor tissue and the blood of patients who undergoing initial surgery. Because this assay is done by a sensitive PCR method, no additional tumor tissue is needed beyond what is removed at the time of surgery. 20 ml of Plasma from patients will be collected at the time of surgery. One hundred patients will be examined the expectation being that 30% will be positive. Correlation will be made between the blood and tissue assay. The details of this protocol including the consent are in the Appendix.

The second protocol entitled, "Estimation of abnormal breast cancer DNA in patient's blood using a sensitive PCR based assay" asks patients for a single plasma sample at the time of routine blood drawing. The rationale behind this protocol is that patient's with widely metastatic cancer have a higher chance of having large amounts of tumor derived DNA in the plasma than newly diagnosed patients with only mammographic lesions. Again only a single sample of plasma will be taken from patients at the time of routine blood drawing.

For both protocols informed consent will be obtained and data will be stored under serial numbers with the master list locked in the principal investigators office. Full information will be available to the DOD.

Key Research Accomplishments

- 5-Aza-Cdr has been shown to induce expression of p16 protein in breast cancer cell lines containing methylated p16 DNA sequences.

- 5-Aza-Cdr also has been demonstrated to cause demethylation of p16 DNA in breast cancer cell lines, consistent with its ability to promote p16 expression by acting as a methylation inhibitor.

- 5-AzaCdr treatment abrogates the tumorigenic property of anchorage-independent growth in breast cancer cell lines. This may result from effects on gene expression in addition to induction of p16 protein.

- Methylated p16 sequences are readily detectable in DNA isolated from plasma of breast cancer patients, potentially providing a non-invasive method for determining p16 methylation status in breast cancer.

Reportable Outcomes

There have been no reportable outcomes to date.

Conclusions

Our studies of the effects of 5Aza-Cdr on p16 gene methylation and p16 protein expression in breast cancer cell lines have shown that this drug effectively blocks p16 gene methylation and induces p16 protein production. 5-Aza-Cdr also blocks anchorage-independent growth of breast cancer cell lines, indicating that treatment with this drug abrogates the tumorigenic properties of these cells. However, control experiments employing breast cancer cell lines with deleted p16 genes, or which fail to express Rb, also lose the ability to grow in an anchorage-independent manner when treated with 5-Aza-Cdr, indicating that effects on gene expression other than the induction of p16 may contribute to this effect. Therefore, a better model for the action of the drug is needed. To accomplish this goal, we are currently screening a number of breast cancer cell lines to find ones that have both methylated p16 and can produce tumors in nude mice to test the efficacy of the drug in a more physiological situation.

Our development of a protocol for detecting methylated p16 sequences in plasma DNA from breast cancer patients provides a potential non-invasive procedure for determining the p16 methylation status of breast cancer patients. These results have led us to propose and design a clinical protocol to determine whether plasma DNA measurements of methylated p16 sequences are a reliable indicator of the p16 methylation status of breast tumor DNA. This methodology may allow investigation of p16 DNA methylation in patients with advanced metastatic breast cancer, since many of these patients are not suitable for biopsy procedures.

Our clinical trial with bryostatin 1 has been put on hold due to poor record keeping and not patient related activities. When the suspension was listed all protocols were resubmitted to the local IRB for reapproval. This process has been completed for the bryostatin-1 protocol and we expect the trial to begin again soon.

"So What" Section

We believe that by combining clinical and basic science studies we can identify new therapies for patients with late stage breast cancer. For example, we are developing model systems to determine the efficacy of methylation inhibitors. These inhibitors could be used in the clinic in patients that have methylated p16 and we are developing a blood test to identify these patients.

Relationship to Statement of Work

Tasks 1, 2 and 3. To implement and evaluate a Phase II clinical trial designed to test the chemotherapeutic efficacy of bryostatin 1 in Stage IV breast cancer patients who have failed high dose chemotherapy.

As described in the previous progress report, this clinical trial has been initiated and is currently ongoing. Evaluation of patients accrued to date has also been carried out, as previously described, with the exception of measurements of p21 expression and cdk activity, since no patients have yet been accrued who are suitable for biopsy procedures. The current status of the bryostatin clinical trial is described in Section VIII of Results.

Task 4. To determine the effects of 5,6-dihydro5-azacytidine (DHAC) on p16 levels and on growth and tumor formation by breast cancer cells.

As described in part in the previous progress report, and confirmed by further studies carried out in the current grant period, DHAC proved to be unsuitable as a DNA methylation inhibitor capable of inducing expression of p16 in breast cancer cell lines. We therefore turned to the more widely studied (but potentially more toxic) methylation inhibitor 5'-aza-2'-deoxycyctidine (5-Aza-Cdr), which has been directly shown to induce expression of p16 protein in several human tumor cell lines containing hypermethylated p16 gene sequences. As described in Sections I and II of Results, we have shown that 5-Aza-Cdr causes demethylation of p16 DNA and expression of p16 protein in breast cancer cell lines, with concomitant loss of growth of these cells in soft agar. However control experiments indicate the expression of other gene products as a result of 5'-Aza-Cdr DNA demethylation can also contribute to the loss of tumorigenic properties in breast cancer cells.

Tasks 6, 7 and 8 To design, implement and evaluate a Phase II clinical trial of DHAC in breast cancer patients who have failed high dose chemotherapy and whose tumors contain methylated p16 DNA.

Due to the previously reported findings that DHAC is unsuitable as a DNA methylation inhibitor capable of inducing p16 in breast cancer cell lines, and the time needed to explore and optimize the use of the alternative drug, 5-Aza-Cdr, the design and implementation of this clinical trial has been delayed. We currently expect that this will occur in year three of the grant.

Because our experience has indicated that few patients at the stage of breast cancer which makes them appropriate candidates for these trials have malignant tissue suitable for biopsy, we have added the development of methodology that will allow the p16 methylation status of breast cancer patients to be determined on plasma DNA. As described below, this has now been included as Task 9 in a Revised Statement of Work.

Revised Statement of Work

In view of the results obtained in the first two years of the project, we propose the following revisions to the original Statement of Work

Tasks 6, 7 and 8 — To design, implement and evaluate a Phase II clinical trial of 5-Aza-Cdr in breast cancer patients who have failed high dose chemotherapy and whose tumors contain methylated p16 DNA.

Because of our results in preclinical studies described above indicating that DHAC is unsuitable as a DNA methylation inhibitor capable of inducing expression of p16 in breast cancer cell lines, we plan to carry out the originally proposed clinical trial of methylation inhibitors using 5-Aza-Cdr instead of DHAC.

Task 9. To develop methodology for determining the p16 methylation status of breast cancer patients by measurements on plasma DNA, and to employ this methodology for the selection of patients for clinical trials of 5-Aza-Cdr in breast cancer. Months 21-36.

As described, in Sections IV - VII of Results, this methodology has been developed and shown to be capable of identifying breast cancer patients whose plasma contains methylated p16 DNA. We have designed and are awaiting approval of clinical protocols (**See Appendix**) that will establish whether detection of plasma methylated p16 DNA is a reliable indicator of the methylation status of breast tumor p16 DNA in patients. Following the establishment of this methodology as a means of selecting appropriate candidate patients, a clinical trial of 5-Aza-Cdr will be designed and implemented, as described in the revised Statement of Work for Tasks 6, 7 and 8 above.

This methodology will also be used in retrospective studies to provide both diagnostic and prognostic information to patients with breast cancer. In this regard, the methodology may provide a useful biomarker to monitor breast cancer progression and remission in patients undergoing conventional therapy.

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Appendix

Figures 1 to 6

Table 1

Two Clinical Protocols and Patient Consent Forms:

#00-848: "Estimation of abnormal breast cancer DNA in patient's blood using a sensitive PCR based assay"

#00-849: "Correlation between methylated p16 DNA in the blood and breast cancer tissue of patients"

Figure 1. Induction of p16 expression in breast cancer cell lines following exposure to 5-Aza-CdR.

Logarithmic phase cultures of the T47-D breast cancer cell line were treated with 1 μM 5-Aza-CdR for the days indicated in the figure at 37°C. The HCT-15 colon cancer cell line was employed as a positive control and the MCF-7 breast cancer cell line (which is homozygously deleted for the p16 gene) was employed as negative control for p16 induction, respectively. In addition, DU4475 breast cancer cells (which lack endogenous RB expression) were treated with 5-Aza-CdR as a positive control for p16 induction but negative control for p16 function (in the absence of downstream RB activity). Protein was isolated from both the untreated and treated cells and assayed for p16 expression by western blot. The SV40-transformed HBL-100 cell line that overexpresses endogenous p16 protein was used as a positive control for p16 expression in the western analysis.

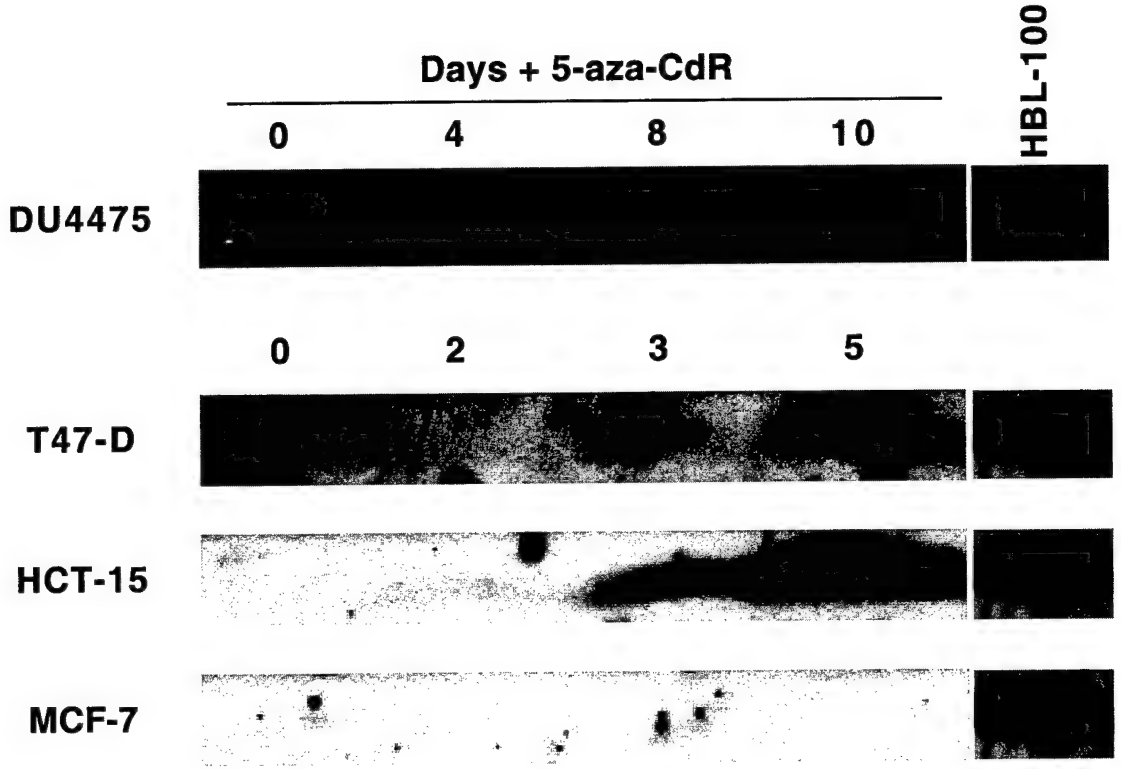
Days + 5-aza-CdR
0
4
8
10
HBL-100
DU4475
0
2
3
5
T47-D
HCT-15
MCF-7

Figure 2. 5-Aza-CdR-mediated demethylation of the p16 gene promoter in breast cancer cell lines.

Logarithmic phase cultures of the T47-D, DU4475, MCF-7 and HCT-15 cancer cell lines were treated with 1 μM 5-Aza-CdR as described in the legend to figure 1. DNA was isolated from both the untreated and treated cells and analyzed for the presence of unmethylated or methylated sequences within the p16 promoter by methylation-specific PCR. The DNA was first modified with sodium bisulfite then subjected to PCR analysis using primers specific for either wildtype (W), methylated (M) or unmethylated (U) p16 promoter sequences. The resulting PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. The lanes labeled 'Blank' contained all of the PCR components minus DNA as a control for contamination.

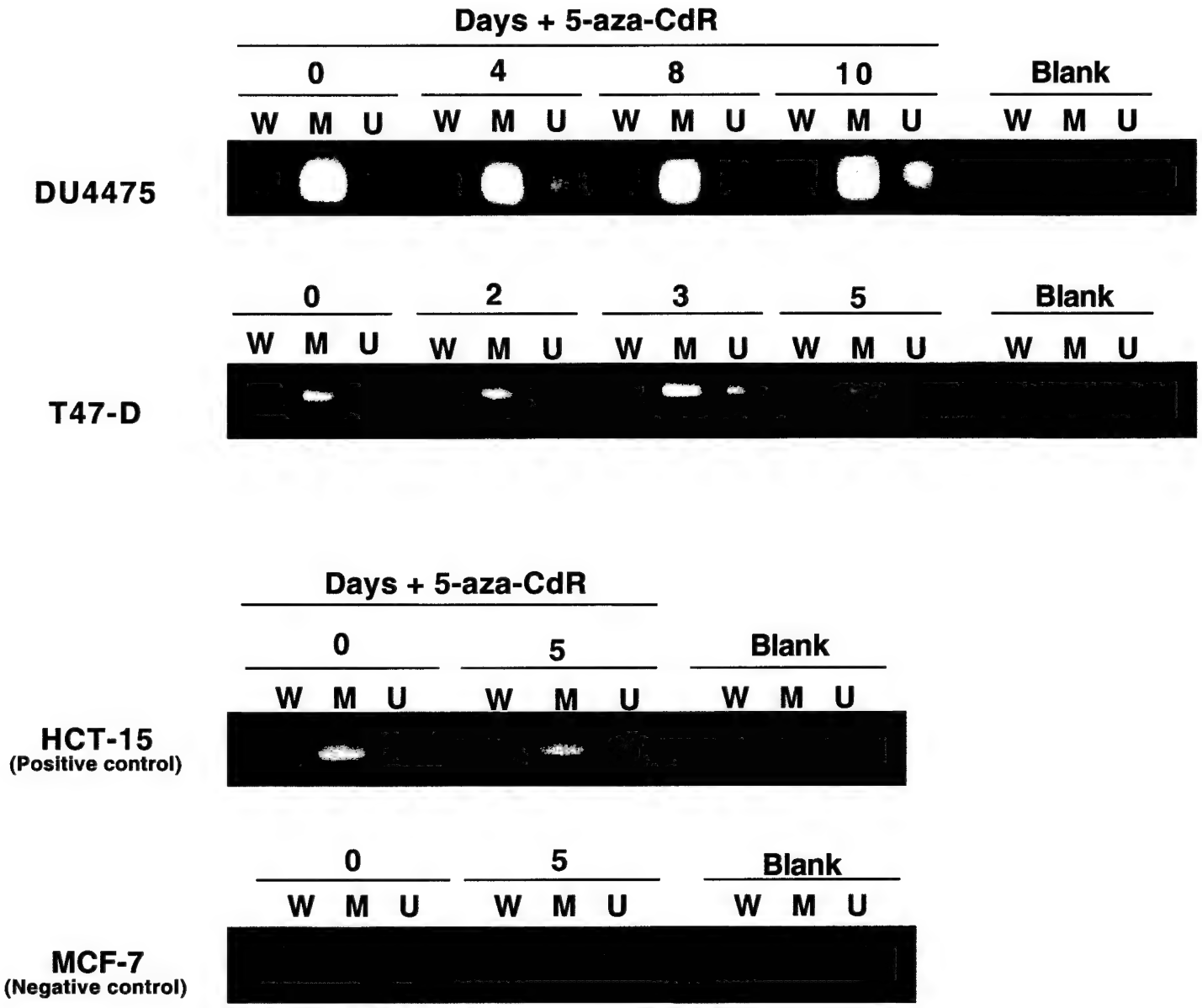
Days + 5-aza-CdR
0
4
8
10
Blank
W M U W M U W M U W M U W M U
DU4475
0
2
3
5
Blank
W M U W M U W M U W M U W M U
T47-D
Days + 5-aza-CdR
0
5
Blank
W M U W M U W M U
HCT-15
(Positive control)
0
5
Blank
W M U W M U W M U
MCF-7
(Negative control)

Figure 3. 5-Aza-CdR inhibits the ability of breast cancer cell lines to grow in soft agar.

T47-D, DU4475 (RB-negative) and MCF-7 (p16-negative) breast cancer cell lines were grown in the presence or absence of 1 μM 5-Aza-CdR for 4 days at 37°C then harvested for p16 expression analysis by western blot (far left hand panels) and growth in soft agar (remaining panels). Cells grown in the absence of 5-Aza-CdR (-AZA) were then plated onto soft agar dishes lacking 5-Aza-CdR while those grown in the presence of 5-Aza-CdR (+AZA) were plated onto soft agar dishes that either lacked or contained 1 μM 5-Aza-CdR and incubated at 37°C. After approximately 14 days, representative dishes from each set of treatments were photographed.

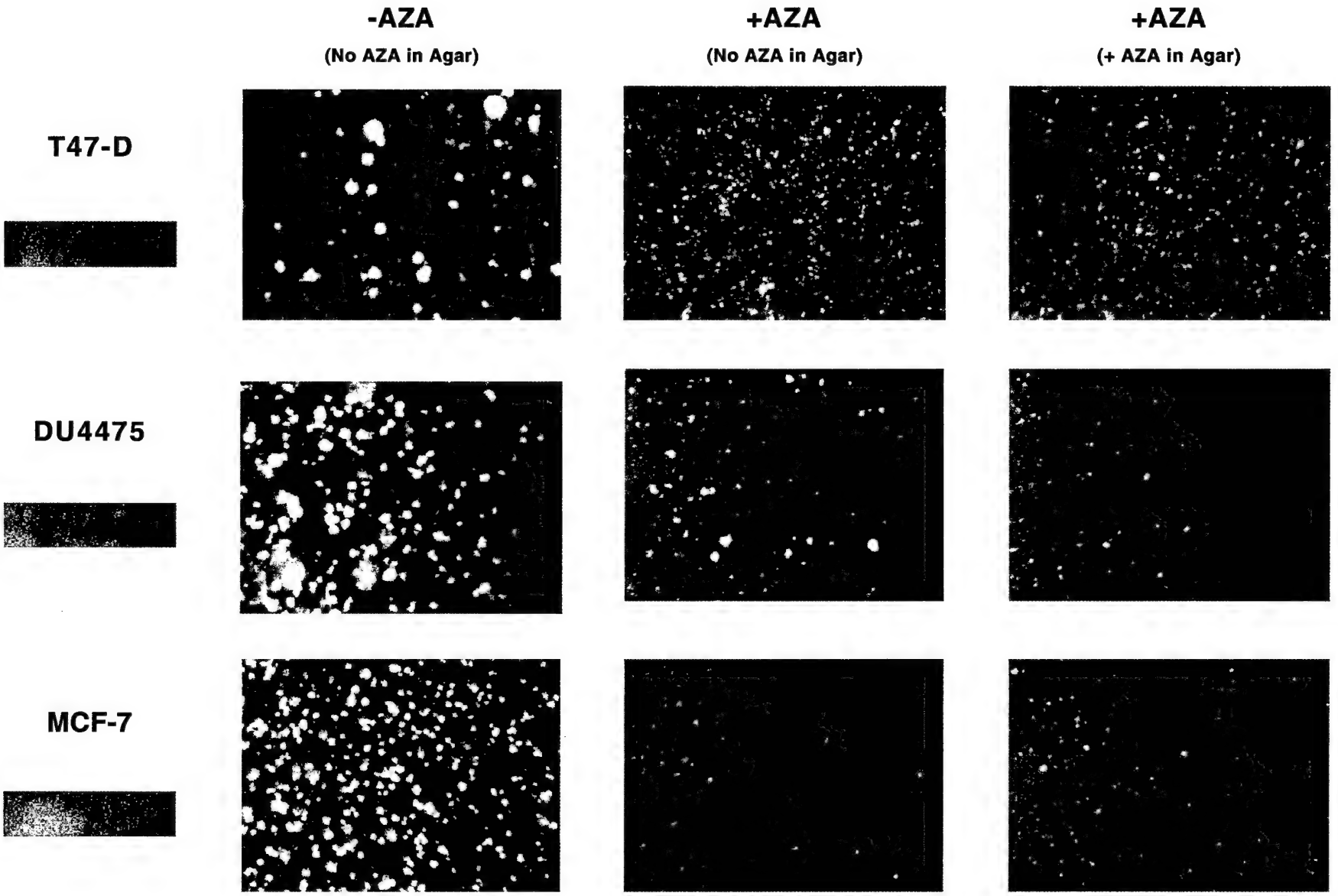
-AZA
(No AZA in Agar)
+AZA
(No AZA in Agar)
+AZA
(+ AZA in Agar)
T47-D
DU4475
MCF-7

Figure 4. Methylation-specific PCR analysis of normal blood plasma DNA at the p16 locus.

10 ml of plasma (previously frozen at –80°C) were thawed then heated to 99°C for 5 minutes. Following centrifugation at 14K rpm, DNA was extracted from the supernatant using a QIAamp blood and tissue kit (Qiagen). 20 µl and 40 µl aliquots of DNA were subjected to sodium bisulfite modification followed by methylation-specific PCR using primers to wildtype (W), methylated (M) and unmethylated (U) p16 promoter sequences. DNA isolated from the breast cancer cell lines, DU4475 and MDA-MB-231, were employed as methylated and unmethylated controls, respectively. The resulting PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. The lanes labeled 'Blanks' contained all of the PCR components minus DNA to control for contamination.

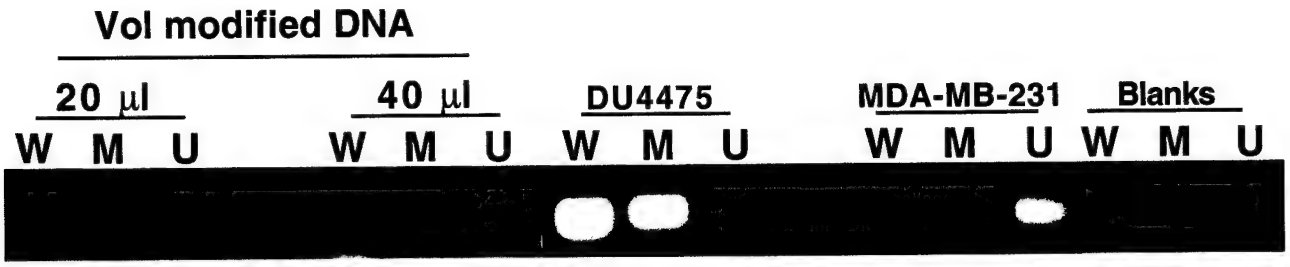
Vol modified DNA
20 µl
40 µl
DU4475
MDA-MB-231
Blanks
W M U W M U W M U W M U W M U

Figure 5. Detection of methylated p16 DNA in plasma.
10 ml aliquots of frozen plasma were thawed and mixed with 0, 10, 20, 50, 100, 250 or 500 ng of HCT-15 colon cancer cell line DNA in which the p16 promoter was homozygously methylated. After thoroughly mixing, DNA was isolated from the plasma samples and subjected to MSP analysis. DNA derived from the HCT-15 cell line was employed as a control for methylated p16 while that derived from a T47-D breast cancer cell line subclone was employed as a control for both methylated and unmethylated p16. The resulting PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. The lanes labeled 'Blank' contained all of the PCR components minus DNA to control for contamination.

Methylated DNA Titration

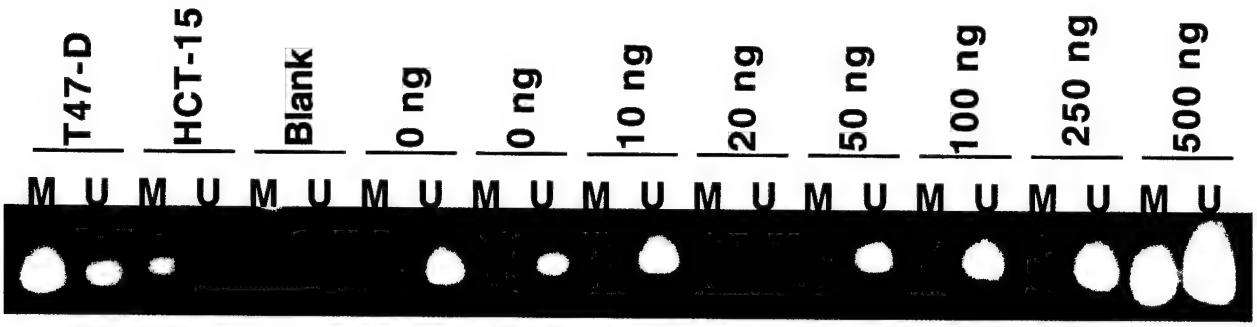

Figure 6. Detection of methylated p16 DNA in plasma from breast cancer patients. 2 ml aliquots of frozen plasma from seven breast cancer patients #1-7) were thawed and subjected to MSP analysis. DNA derived from a T47-D breast cancer cell line subclone was employed as a control for both methylated and unmethylated p16 (see Figure 4). The resulting PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. The lanes labeled 'Blank' contained all of the PCR components minus DNA to control for contamination.

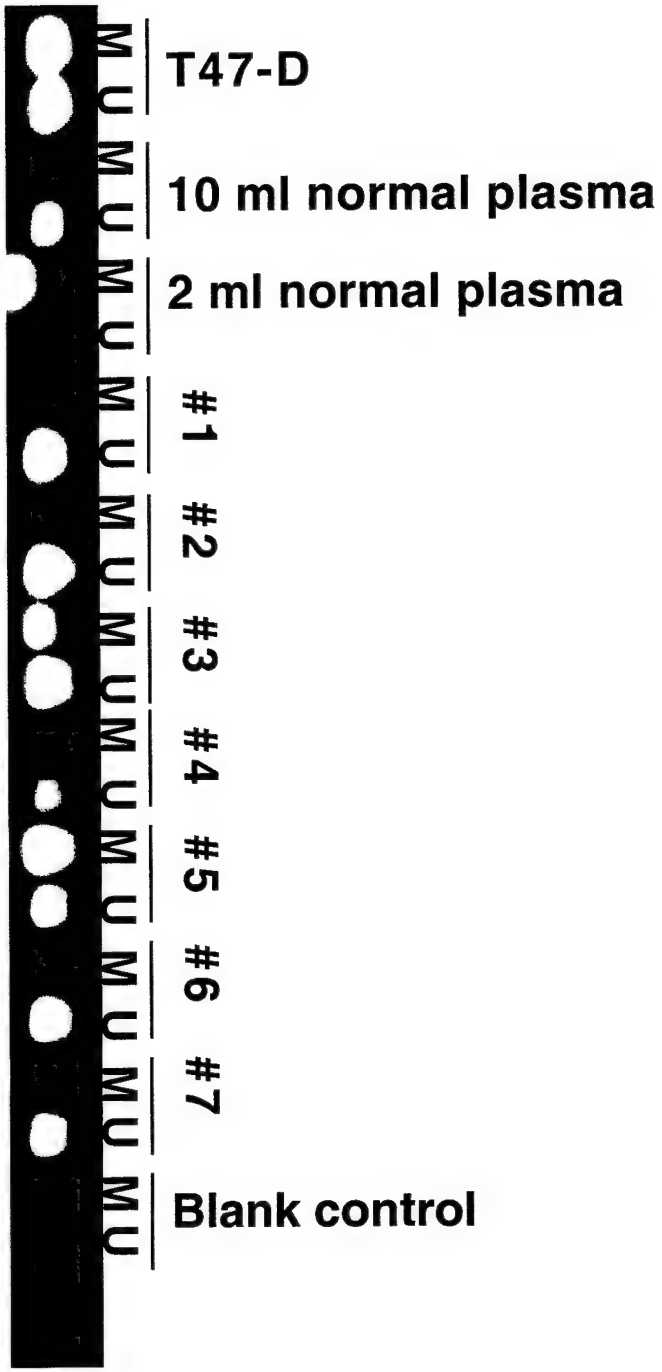
T47-D
10 ml normal plasma
2 ml normal plasma
#1
#2
#3
#4
#5
#6
#7
Blank control

Table 1. Effect of 5-Aza-CdR treatment upon the ability of breast cancer cell lines to form colonies in soft agar.

Cell Line	5-aza-CdR Treatment	No. Cells Plated	Mean No. Colonies
T47-D	-Cells -Agar	$2.5x10^5$	25,280
	+Cells +Agar	$2.5x10^5$	3
	+Cells -Agar	$2.5x10^5$	4
DU4475	-Cells -Agar	$2.5x10^5$	12,992
	+Cells +Agar	$2.5x10^5$	2
	+Cells -Agar	$2.5x10^5$	648
MCF-7	-Cells -Agar	$2.5x10^5$	20,416
	+Cells +Agar	$2.5x10^5$	0
	+Cells -Agar	$2.5x10^5$	4

Protocol Summary

Project Title: Estimation of abnormal breast cancer DNA in patient's blood using a sensitive PCR based assay.

Investigators: Andrew S. Kraft, MD, Christina A. Finlayson, MD, Robert Sclafani, Ph.D. and Thomas A. Langan, Ph.D.

COMIRB# 00-848

Date: August 28th, 2000 Revised October 6th, 2000

Background/Rationale

The importance of defects in the Rb pathway in the development of cancer is indicated by the fact that almost all cancer cells are defective in some aspect of its regulation (1-5). This near universal prevalence of defects in components of the Rb pathway suggests that overcoming normal cell cycle regulation at this point is necessary condition for the development of malignancy (5). These defects include loss of expression of Rb (6,7), overexpression of cyclin D1 (8), and loss of expression of p16 inhibitor protein, whose role as a tumor suppressor is now well documented (9). In particular, the importance of p16 in tumor suppression is seen in that homozygous p16 deletions in mice result in spontaneous development of multiple tumor types (10).

Recognition of the importance of p16 as a tumor suppressor comes in part from the recent discovery that gene methylation, in addition to homozygous deletion or loss of heterozygosity seen for other tumor suppressor genes is a major mechanism for inactivation of p16 gene in all common human cancers (11,12). In primary breast tumors, which only rarely show homozygous p16 deletions or point mutations (13,14), 31% of tumors were found to contain p16 genes inactivated by methylation (11). We hypothesize that p16 gene methylation is responsible for uncontrolled cellular proliferation in many breast tumors, and that agents that increase this protein will prevent the growth of cancer. For example, DHAC, an NCI-produced (NSC #264880) methylation inhibitor has been tested in phase I and phase II clinical trials.

It has been known for several years that the plasma component of circulating blood contains tiny quantities of free DNA. The concentration of DNA in the plasma of healthy individuals is approximately 14 ng/ml DNA'. This level increases significantly in individuals diagnosed with different types of cancer to approximately 180 ng/ml2.

The ability to detect tumor-specific molecular defects in the circulating blood would obviate the requirement for tumor biopsy material thus providing a more efficient and noninvasive means of screening for a vast array of molecular aberrations. Our objective is to identify women whose tumors contain methylated pl6 genes for recruitment into a clinical trial that aims to test the efficacy of therapeutic demethylating

agents. We therefore propose to determine the feasibility of assaying plasma DNA for the presence of methylated pl6 alleles in women diagnosed with metatstatic breast cancer. DNA will be extracted from circulating blood plasma then assayed for pl6 promoter methylation status by methylation-specific PCR (MSP)3. The normal breast tissue is expected only to contain unmethylated pl6 whereas the tumor tissue may contain either unmethylated or methylated pl6. If the tumor DNA contains methylated pl6 alleles, we should also expect to see methylated pl6 in the plasma DNA in addition to normal cell-specific unmethylated pl6.

Hypothesis

We hypothesize that a PCR based assay method can be used to detect methylated p16 gene in the blood of breast cancer patients. This test will replace the necessity of obtaining breast cancer tissue to measure the methylation of this gene.

Purpose

The data from this study will determine the utility of this PCR based blood test in detecting methylated or unmethylated p16 DNA in the blood of breast cancer patients. Since 30% of breast cancer patients are expected to have methylated p16 DNA in their tumors, the data collected in this study will begin to evaluate the sensitivity of this blood test.

Methods

Women being evaluated in breast cancer clinic who are found to have metastatic disease will be asked to sign a consent allowing us to obtain a blood sample (20 ml). All data concerning the extent of disease and disease progression will be kept confidential. The patient will be identified by number only and the data will be kept in locked files.

• Isolation of DNA from blood plasma

Using 10 ml plasma (from approximately 20 ml whole blood collected in EDTA containing tubes), we have optimized the following isolation procedure to yield sufficient DNA for an initial MSP assay plus three further confirmatory reactions.

The 10 ml plasma sample (which may be frozen at -80•C prior to analysis) is first heated at 99•C for 5 minutes then centrifuged at high speed to allow recovery of the clear, DNA containing, supernatant. Following an overnight incubation with proteinase K the DNA is purlfied on QIAamp columns (Qiamp Blood Kit; Qiagen Inc., Hilden, Germany) according to the Blood and Body Fluids protocol.

• Methylation-specific PCR analysis of plasma

The total plasma DNA sample is then used for the MSP assay. The DNA is first modified overnight with sodium bisulfite which converts only unmethylated cytosines to uracil. Following purification on Wizard columns (Promega, Inc.), the DNA is precipitated with ammonium acetate and ethanol. One-tenth of the resulting DNA is then subjected to PCR analysis using oligonucleotides designed from the promoter of the pl6 gene that are specific for wild type (unmodified), methylated and unmethylated DNA sequences .

We have performed MSP analysis of multiple independently-isolated normal plasma DNA preparations and, as expected, shown amplification using the unmethylated DNA-specific oligonucleotides only. Furthermore, we have titrated cell line DNA containing methylated pl6 alleles into plasma samples prior to isolation of plasma DNA and shown that we can successfully amplify as little as 12.5 ng methylated DNA in addition to the unmethylated normal plasma DNA using this method. Because the normal plasma DNA contains only unmethylated DNA, this system will enable us to identify tumor-specific methylated DNA in the plasma of breast cancer patients.

Observations

1- We would plan to determine that the above assay could be done on actual patient material.
2- Since approximately 30% of the tumors are positive to p16 methylation, we would plan to test whether the assay picks up this number of tumors.
3- We would plan to correlate the stage and extent of disease with the number of positive assays.

Inclusion criteria

Woman ages 18-72 years that are known to have metastatic breast cancer as determined by x-ray and clinical history.

Exclusion criteria

1- Patients deemed not competent to make their own decision
2- Patients with a previous history of another cancer.

Sample Size

The sample size will be 100 patients. We will be able to establish a confidence interval of at least ±0.1on the blood samples with a sample size of 100. This can be established regardless of the sensitivity or specificity of the assay in either case. We will be able to provide a similar confidence interval for the proportion detected by the blood test and see if that covers the anticipated 30% prevalence of methylation.

Estimated Duration of Study

The estimated duration of the study is 1 year. If the samples are collected more quickly the study will be terminated.

Examinations and evaluations

Other than the drawing of 20 cc of blood no further examination of the patient will be done. The extent of metastatic breast cancer will be noted from the chart.

Drugs, devices or instruments

No novel drugs devices or instruments are involved in this study.

Data analysis

The data analysis will be carried out by Dr. J. Murphy in biostatistics.

Data Security

All electronic data will be secured in protected files. All paperwork will be secured under lock and key. The data will be coded and the master file placed in a locked file cabinet in the Principal Investigators office.

Changes from Usual Treatment

There will be no changes in treatment. All participants will receive therapy deemed as standard of care by their primary oncologist.

Risks

Subject: None above the standard risks involved with standard blood draw.

Investigators: Handling of blood products.

Benefits

None

Funding

The evaluation of blood tests is funded by the Department of Defense Breast Cancer Research Program.

Special Consent issues

The research protocol and the purpose of the study will be explained to each study participant. The consent will be obtained by the primary investigaotr, the co-investigators, or a clinical nurse in the cancer center on behalf of the investigators. All of the investigators getting consent will either have COMIRB training or been trained by a Principal investigator who has completed COMIRB training.

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14. Quesnel, B., Fenaux, P., Philippe, N., Fournier, J., Bonneterre, J., Preudhomme, C., and Peyrat, J.P. Analysis of p16 gene deletion and point mutation in breast carcinoma. Br. J. Cancer, 72: 351-353, 1995.

Consent Form Approval

Allan Prochazka, MD/Stephen Bartlett, R.Ph., Co-Chairs, COMIRB
Christopher Kuni, MD/Ken Easterday, R.Ph., Co-Chairs, COMIRB
Adam Rosenberg, MD/David Lawellin, Ph.D..,Co-Chairs, COMIRB

Date: Valid Through:_____

COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD

"Estimation of abnormal breast cancer DNA in patient's blood using a sensitive PCR based assay"

Principal Investigator: Andrew S. Kraft
SUBJECT CONSENT FORM
September 6, 2000/COMIRB Protocol Number 00-848

Project Description

You are being asked to take part in an investigational study evaluating the use of a blood assay to measure the levels of a specific modified piece of DNA in your blood. Breast cancer is caused in part by the lack of the ability of cells to produce specific proteins that inhibit the growth of cells. This lack of protein production is caused by modification of cellular DNA. This DNA abnormality is not an inherited trait and the reason it occurs is not known. Since tumor cells are growing and dying some of this abnormal DNA may be released into the blood. Using a newly developed laboratory assay, we would like to attempt to measure this abnormal DNA in your blood. One hundred women with metastatic breast cancer will be enrolled into this study. Participation in this study is voluntary and the purpose of this consent is to inform you about the study and its possible benefits and risks.

Procedures

If you agree to participate, you will need to donate a single 20ml of blood (two tablespoons) at a time when you are known to have breast cancer. This blood will be sent to the lab for evaluation of abnormal DNA levels. The principal investigator or a designee will review your chart in order to identify information regarding the stage of your breast cancer.

Initials _____

Discomforts and Risks

Venipuncture Risk
Approximately 2 tablespoon of blood (20ml) will be removed by putting a needle into your vein at the time of your pre-operative evaluation. This is the standard method used to obtain blood for tests. You will feel pain when the needle goes into the vein. A bruise may form at the site.

Benefits
You will receive no benefit from participating in this research study and there are risks as mentioned in the risk section.

Source of Funding
All funding for this study will be provided by the Army Medical Research Command.

Cost to Subject
There is no cost to you for participating in this study. You will not be paid for your participating in this study.

Study Withdrawal
You may choose not to enter the study or withdraw from the study at any time and your doctor will continue to take care of you without loss of benefits to which you are entitled. Significant new findings that relate to your participation in this study will be discussed with you.

Invitation for Questions
You will receive a copy of this consent form. Please ask questions about this research or consent either now or in the future. You may direct your questions to Dr. Andrew S. Kraft, MD 303-315-8802. If you have questions regarding your rights as a research subject, please call the Colorado Multiple Institutional Review Board (COMIRB) office at (303) 724-1055.

Confidentiality
Your physician/investigator and the Army Breast Cancer Research Command will treat your identity with professional standards of confidentiality. However, the U.S. Department of Health and Human Services, and the Colorado Multiple Institute Review Board have the right to inspect all of your medical records relating to this research for the purpose for verifying data. The information obtained in this study may be published in medical journals, but your identity will not be revealed.

Injury and Compensation
If you are hurt by this research, we will provide medical care if you want it, but you will have to pay for the care that is needed. You will not be paid for any other loss as a result of the injury, such as loss of wages, pain and suffering. Further information can be obtained by calling Andrew S. Kraft, MD 303-315-8802.

Initials _____

AUTHORIZATION:

I have read this paper about the study or it was read to me. I know what will happen, both the possible good and bad (benefits and risks). I choose to be in this study. I know I can stop being in this study and I will still get the usual medical care. I will get a copy of this consent form. (Initial all the previous pages of this consent form)

Signature: _____ Print Name _____ Date _____
Subject

Consent form explained by: _____ Print Name _____ Date _____

Investigator _____ Date _____

Initials _____

Project Title: Correlation between methylated p16 DNA in the blood and breast cancer tissue of patients.

Investigators: Andrew S. Kraft, MD, Christina A. Finlayson, MD, Robert Sclafani, Ph.D. and Thomas A. Langan, Ph.D.

COMIRB# 00-849

Date: August 28th, 2000 Revised October 5, 2000

Background/Rationale

The importance of defects in the Rb pathway in the development of cancer is indicated by the fact that almost all cancer cells are defective in some aspect of its regulation (1-5). This near universal prevalence of defects in components of the Rb pathway suggests that overcoming normal cell cycle regulation at this point is necessary condition for the development of malignancy (5). These defects include loss of expression of Rb (6,7), overexpression of cyclin D1 (8), and loss of expression of p16 inhibitor protein, whose role as a tumor suppressor is now well documented (9). In particular, the importance of p16 in tumor suppression is seen in that homozygous p16 deletions in mice result in spontaneous development of multiple tumor types 10).

Recognition of the importance of p16 as a tumor suppressor comes in part from the recent discovery that gene methylation, in addition to homozygous deletion or loss of heterozygosity seen for other tumor suppressor genes is a major mechanism for inactivation of p16 gene in all common human cancers (11,12). In primary breast tumors, which only rarely show homozygous p16 deletions or point mutations (13,14), 31% of tumors were found to contain p16 genes inactivated by methylation (11). We hypothesize that p16 gene methylation is responsible for uncontrolled cellular proliferation in many breast tumors, and that agents that increase this protein will prevent the growth of cancer. For example, DHAC, an NCI-produced (NSC #264880) methylation inhibitor has been tested in phase I and phase II clinical trials.

It has been known for several years that the plasma component of circulating blood contains tiny quantities of free DNA. The concentration of DNA in the plasma of healthy individuals is approximately 14 ng/ml DNA'. This level increases significantly in individuals diagnosed with different types of cancer to approximately 180 ng/ml.

The ability to detect tumor-specific molecular defects in the circulating blood would obviate the requirement for tumor biopsy material thus providing a more efficient and noninvasive means of screening for a vast array of molecular aberrations. We propose to determine the feasibility of assaying plasma DNA for the presence of methylated pl6 alleles in women diagnosed with breast cancer. DNA will be extracted from circulating blood plasma then assayed for pl6 promoter methylation status by methylation-specific PCR (MSP)3. The normal breast tissue is expected only to contain

unmethylated pl6 whereas the tumor tissue may contain either unmethylated or methylated pl6. If the tumor DNA contains methylated pl6 alleles, we should also expect to see methylated pl6 in the plasma DNA in addition to normal cell-specific unmethylated pl6. Normal and tumor tissue will be obtained at the time of operation or biopsy. No additional tissue will be examined other than that removed at the time of surgery for routine pathology.

This study comparison study of blood and tissue will help us to verify this assay.

Hypothesis

We hypothesize that there will be a strict correlation between the occurrence of methylated p16 in tumor samples and the blood of patients. This result would validate the use of a PCR based assay method to detect methylated p16 gene in the blood of breast cancer patients. This test will replace the necessity of obtaining breast cancer tissue to measure the methylation of this gene. In the future this assay may have used in designing new treatment stategies, although no clinical protocols are currently planned.

Purpose

The data from this study will determine the correlation between breast tissue methylation of the p16 gene and the blood of patients evaluated using a PCR based blood test. Since 30% of breast cancer patients are expected to have methylated p16 DNA in their tumors, the data collected in this study will evaluate the sensitivity and specificity of this blood test.

Methods

Women being evaluated in breast cancer clinic who are found to have breast cancer metastatic disease will be asked to sign a consent allowing us to obtain a blood sample (20 ml) prior to operation or breast biopsy. A small amount of tissue from the surgery that is discarded at the time of routine pathology will be analyzed in the laboratory . All data concerning the extent of disease and disease progression will be kept confidential.

• Isolation of DNA from blood plasma

Using 10 ml plasma (from approximately 20 ml whole blood collected in EDTA containing tubes), we have optimized the following isolation procedure to yield sufficient DNA for an initial MSP assay plus three further confirmatory reactions.

The 10 ml plasma sample (which may be frozen at -80•C prior to analysis) is first heated at 99•C for 5 minutes then centrifuged at high speed to allow recovery of the clear, DNA containing, supernatant. Following an overnight incubation with proteinase K

the DNA is purlfied on QIAamp columns (Qiamp Blood Kit; Qiagen Inc., Hilden, Germany) according to the Blood and Body Fluids protocol.

• Isolation of DNA from normal and tumor tissues

5-10 mg finely minced tissues are incubated 55•C in lysis buffer containing proteinase K until the tissue is completely lysed. The DNA is then purlfied on QIAamp columns (QIAamp Tissue Kit; Qiagen Inc., Hilden, Germany) according to the Tissue protocol.

• Methylation-specific PCR analysis of plasma or tumor samples.

Two micrograms of tissue DNA or one-fourth of the total plasma DNA sample is then used for the MSP assay. The DNA is first modified overnight with sodium bisulfite which converts only unmethylated cytosines to uracil. Following purification on Wizard columns (Promega, Inc.), the DNA is precipitated with ammonium acetate and ethanol. One-tenth of the resulting DNA is then subjected to PCR analysis using oligonucleotides designed from the promoter of the pl6 gene that are specific for wild type (unmodified), methylated and unmethylated DNA sequences [3].

We have performed MSP analysis of multiple independently-isolated normal plasma DNA preparations and, as expected, shown amplification using the unmethylated DNA-specific oligonucleotides only. Furthermore, we have titrated cell line DNA containing methylated pl6 alleles into plasma samples prior to isolation of plasma DNA and shown that we can successfully amplify as little as 12.5 ng methylated DNA in addition to the unmethylated normal plasma DNA using this method. Because the normal plasma DNA contains only unmethylated DNA, this system will enable us to identify tumor-specific methylated DNA in the plasma of breast cancer patients.

Observations

1- We will plan to determine that the above assay could be done on actual patient material.
2- Since approximately 30% of the tumors are positive to p16 methylation, whether the results of this blood assay correlates with the tumor samples.
3- We would plan to correlate the stage and extent of disease with the number of positive assays in the blood.

Inclusion criteria

Woman ages 18-72 years that are known to have breast cancer and are set to undergo biopsy or operation as determined by x-ray and clinical history.

Exclusion criteria

1- Patients deemed not competent to make their own decision

2- Patients with a previous history of another cancer.

Sample Size

We will be able to establish a confidence interval of at least ±0.1on the percent agreement of the paired tissue and blood samples with a paired sample size of 100. This estimate of the level of agreement can be established regardless of the sensitivity or specificity of the assay in either case.

Estimated Duration of Study

The estimated duration of the study is 1 year. If the samples are collected more quickly the study will be terminated.

Examinations and evaluations

Other than the drawing of 20 cc of blood no further examination of the patient will be done. The extent of breast cancer will be noted from the chart.

Drugs, devices or instruments

No novel drugs devices or instruments are involved in this study.

Data analysis - Data analysis will be carried out by Dr. Jim Murphy in biostatistics.

Data Security

All electronic data will be secured in protected files. All paperwork will be secured under lock and key. The patient will be identified by number only and the data will be kept in locked files in the prinicple investigators office.

Changes from Usual Treatment

There will be no changes in treatment. All participants will receive therapy deemed as standard of care by their primary oncologist. Because of the extreme sensitivity of this PCR technique no additional normal or malignant breast tissue will be removed.

Risks

Subject: None above the standard risks involved with standard blood draw.

Investigators: Handling of blood products and human tissue.

Benefits

None

Funding

The evaluation of blood tests is funded by the Department of Defense Breast Cancer Research Program.

Special Consent issues

The research protocol and the purpose of the study will be explained to each study participant. The consent will be obtained by the primary investigaotr, the co-investigators, or a clinical nurse in the cancer center on behalf of the investigators. All those consenting patients will have COMIRB training or will be trained by someone who has completed the COMIRB course.

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Consent Form Approval

Allan Prochazka, MD/Stephen Bartlett, R.Ph., Co-Chairs, COMIRB
Christopher Kuni, MD/Ken Easterday, R.Ph., Co-Chairs, COMIRB
Adam Rosenberg, MD/David Lawellin, Ph.D..,Co-Chairs, COMIRB

Date: Valid Through:_____

COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD

"Correlation between methylated p16 DNA in the blood and breast cancer tissue of patients"

Principal Investigator: Andrew S. Kraft
SUBJECT CONSENT FORM
September 6, 2000/COMIRB Protocol Number 00-849

Project Description

You are being asked to take part in an investigational study evaluating the use of a blood assay to measure the levels of a specific modified piece of DNA in your blood. Breast cancer is caused in part by the lack of the ability of cells to produce specific proteins that inhibit the growth of cells. This lack of protein production is caused by modification of cellular DNA. This DNA abnormality is not an inherited trait and the reason it occurs is not known. Since tumor cells are growing and dying some of this abnormal DNA may be released into the blood. One hundred women will be enrolled into this study with 20 women each having Stage II, III or IV breast carcinoma. This study will identify whether the abnormal DNA found in your breast cancer is also found in your blood. Participating in this study does not mean that you have any hereditary abnormality in your DNA. This study will measure the levels of abnormal DNA found in your tumor and attempt to correlate the results of this blood test with the tumor sample. Participation in this study is voluntary and the purpose of this consent is to inform you about the study and its possible benefits and risks.

Procedures

If you agree to participate, you will need to donate 20 ml of blood (two tablespoons), at the time of your pre-operative evaluation. This blood will be sent to the lab for evaluation of free DNA. During your surgery your doctor will be removing tissue for clinical evaluation. When the pathologic evaluation is complete, we will take the unused tissue for further study. The tissue to be used for this study would normally be discarded. No additional tissue will be taken at the time of operation The findings in the research lab will then be correlated with the results of the blood test. You will not

Initials _____

receive any information regarding these additional tests. The principal investigator or a designee will review your chart in order to identify information regarding the stage of your breast cancer.

Discomforts and Risks

Breast Biopsy

There will be no additional risk to you other than the usual risks for your surgery. This study will not involve any additional operative procedures. No additional risks are associated with this research since the tissue being used for clinical purposes only.

Venipuncture Risk

Approximately 1 tablespoon of blood (20 ml) will be removed by putting a needle into your vein at the time of your pre-operative evaluation. This is the standard method used to obtain blood for tests. You will feel pain when the needle goes into the vein. A bruise may form at the site.

Benefits

You will receive no benefit from participating in this research study and there are risks as mentioned in the risk section.

Source of Funding

All funding for this study will be provided by the Army Breast Cancer Research Command.

Cost to Subject

There is no cost to you for participating in this study. There will be no charge for procedures or labs required by the study. You will not be paid for your participating in this study.

Study Withdrawal

You may choose not to enter the study or withdraw from the study at any time and your doctor will continue to take care of you without loss of benefits to which you are entitled. Significant new findings that relate to your participation in this study will be discussed with you.

Invitation for Questions

You will receive a copy of this consent form. Please ask questions about this research or consent either now or in the future. You may direct your questions to Dr. Kraft at (303) 315-8802. If you have questions regarding your rights as a research subject, please call the Colorado Multiple Institutional Review Board (COMIRB) office at (303) 724-1055.

Confidentiality

Your physician/investigator and the Army Breast Cancer Research Command will treat your identity with professional standards of confidentiality. However, the U.S. Department of health and Human Services, the Army Research Command and the

Colorado Multiple Institute Review Board have the right to inspect all of your medical records relating to this research for the purpose for verifying data. The information obtained in this study may be published in medical journals, but your identity will not be revealed.

Injury and Compensation

If you are hurt by this research, we will provide medical care if you want it, but you will have to pay for the care that is needed. You will not be paid for any other loss as a result of the injury, such as loss of wages, pain and suffering. Further information can be obtained by calling Andrew S. Kraft, MD 303-315-8802.

AUTHORIZATION:

I have read this paper about the study or it was read to me. I know what will happen, both the possible good and bad (benefits and risks). I choose to be in this study. I know I can stop being in this study and I will still get the usual medical care. I will get a copy of this consent form. (Initial all the previous pages of this consent form)

Signature: _____ Print Name _____ Date _____
Subject

Consent form explained by: _____ Print Name _____ Date _____

Investigator _____ Date _____

Initials _____